RUNNING HEAD: Cisplatin Ototoxicity

Protection of Cisplatin Ototoxicity with a Novel Src Inhibitor

Senior Thesis

Presented in Partial Fulfillment of the Requirements for Graduation "with Research Distinction in Speech and Hearing Science" in the Speech and Hearing Science Department of The Ohio State University

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The Ohio State University, May 2014

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Acknowledgements

The authors thank Ryan Harrison and Riley DeBacker for assistance with study data collection. Research was supported by a Social and Behavioral Sciences Undergraduate Research grant and by a contract with AstraZeneca Pharmaceuticals.

Table of Contents

Abstract

Cisplatin is a drug used to treat a variety of cancers. It induces apoptosis, or programmed cell death in tumor cells, thus reducing tumor size and growth. Although the goal of this chemotherapy drug is to kill harmful cancer cells, it also damages other cells, such as outer hair cells (OHCs), fundamental cells within the inner ear responsible for the sensation of hearing. The intracellular signaling molecule, Src, a protein tyrosine kinase, has been implicated as a major signaling molecule in some cancer cells. In cancer, Src is believed to be hyper-active, and triggers unchecked cell division. In the cochlea, the stress on the cell induced by cisplatin is believed to trigger Src-mediated apoptosis. For this experiment, sixteen rats were tested in order to determine if saracatinib, a Src inhibitor, can prevent cisplatin ototoxicity. Eight rats served as the experimental group, and were exposed to cisplatin along with saracatinib. The other eight rats received the vehicle solution without the saracatinib prior to cisplatin exposure. Hearing sensitivity was measured using the auditory brainstem response and distortion product otoacoustic emissions. All animals were administered the protective compound over the nine days by oral gavages. A dose of 12mg/kg of cisplatin was administered after the second day of the gavages. All animals were retested three days, then six days after the cisplatin treatment to determine if a change in hearing threshold was present.

List of Figures

Introduction

Cancer treatment drugs can be extremely powerful with serious side effects that can significantly change one's life. Cisplatin is one of the many chemotherapeutic drug used to treat a variety of cancers, including testicular, ovarian, basal cell, and head and neck cancer. Although cisplatin is a commonly used drug in treating malignant tumors, it has several serious side effects, including nephrotoxicity, neuropathy, muscle loss, and ototoxicity. Drug ototoxicity is a condition in which the drug induces damage to the cochlea, the sense organ for hearing. Damage to the cochlea results in sensorineural hearing loss (SNHL)(ASHA, 2013). SNHLs are typically permanent and cannot be medically treated. Cisplatin is currently only used in small doses because of the ototoxicity and other serious side effects (Rybak, 2009). There is an ongoing need in the hearing research community to develop supplemental drugs that can prevent the cisplatin dose limitations imposed by the severity of its side effects, most notably ototoxicity. What is crucial for these co-treatment drugs is that they do not reduce or compromise the tumor cell-killing ability of the chemotherapy drug.

Cisplatin kills tumor cells by inducing apoptosis, or programmed cell death. Although the goal of this chemotherapy drug is to kill harmful cancer cells, it also induces apoptosis in cells within the cochlea, such as outer hair cells (OHCs), fundamental cells within the inner ear responsible for the sensation of hearing. When the inner ear is exposed to cisplatin, OHCs at the base of the cochlea are the most susceptible to cisplatin-induced cochlear damage. The inability to discriminate fine frequencies and loss of sensitivity to low- intensity sounds are two negative outcomes that result from the deterioration of

OHCs. As cisplatin doses continue to increase, OHC losses expand into the middle portion of the cochlea, and abnormalities in the stria vascularis, supporting cells, inner hair cells, and spiral ganglion cells become apparent. If the inner hair cells and spiral ganglion cells are damaged, the cochlea will not be able to accurately transmit complex sound signals to the brain, resulting in a loss of speech discrimination (Bielefeld et al., 2013).

Several different drugs, including a variety of antioxidants have been tested as supplemental treatments with cisplatin to avoid ototoxic side effects. Antioxidants are used because cisplatin generates reactive oxygen species (ROS), and depletes antioxidants in the cells that it enters (whether they are tumor cells or cochlea cells). If ROS levels exceed antioxidant levels, there is a potential for oxidative stress, a condition that results in cell and tissue damage, and can act as a trigger for apoptosis. Increasing the amount of antioxidants taken in addition to the cisplatin treatment creates a stronger defense against oxidative stress, preventing damage to cells, such as OHCs. Antioxidants, such as Trolox, D-methionine, and ebselen have been used in previous experiments to prevent cisplatin ototoxicity by enhancing cochlear antioxidant defenses (Bielefeld et al., 2013; Kopke et al., 1997; Jamesdaniel et al., 2012; Campbell et al., 1996; Campbell et al., 1999; Feghali et al., 2001; Fetoni et al., 2004; Lynch et al., 2005; Campbell et al., 2007).

The intracellular signaling molecule, Src, a protein tyrosine kinase, has been implicated as a major signaling molecule in some cancer cells. Src acts by catalyzing a reaction in which a phosphate group is attached to a protein to activate it. In many cells, Src acts as intracellular bridge that communicates environmental changes at the cell's surface to the nucleus of the cell in order to evoke changes in gene expression to either defend against injury or to trigger apoptotic cell death. In cancer, Src is believed to be

hyper-active, and triggers unchecked cell division. In the cochlea, the stress on the cell induced by cisplatin is believed to trigger Src-mediated apoptosis (Bielefeld et al., 2013).

The current project sought to reduce cisplatin ototoxicity using a Src inhibitor, saracatinib. Saracatinib acts both as a potent inhibitor of Src and as a blocker of organic cation transporter (OCT). OCT is a protein on some cells responsible for allowing different positively charged molecules into the cell. Cisplatin uses the OCT as the pathway into the cells in the kidney and the cochlea. Blockade of OCT has been shown to reduce hearing loss and kidney damage from cisplatin (Ciarimboli et al., 2010). In order to prevent damage to the cochlea, the goal for the Src inhibitor is to block the OCT and stop cisplatin from entering the OHCs, and to inhibit Src signaling that would trigger apoptosis in OHCs affected by cisplatin.

Anticipated results

The result anticipated in the study was that saracatinib would prevent apoptosis within the inner ear and preserve OHCs and hearing sensitivity after toxic exposure to cisplatin. Beyond the current study, the hope is that saracatinib could prevent ototoxicity while preserving or enhancing cisplatin's tumor cell-killing potency.

Methods

For this experiment, sixteen Fischer 334/NHsd rats were tested in order to determine if saracatinib can successfully prevent cisplatin ototoxicity. Eight were designated as the experimental group, and were exposed to cisplatin along with the protective Src inhibitor. The Src inhibitor was mixed into a suspension and delivered once per day for nine days by oral gavage. The cisplatin was delivered on the third day of the oral gavage treatments. The other eight rats were control subjects. They received the

suspension vehicle without the saracatinib and also were given the 12 mg/kg dose of cisplatin.

Auditory Assessments

Hearing sensitivity was measured using the auditory brainstem response (ABR) across a range of six frequencies (5, 10, 15, 20, 30, and 40kHz) and distortion product otoacoustic emissions (DPOAEs). Throughout all testing, the animals were anesthetized with inhalant isoflurane, 4% for induction, 1.5% for maintenance, with a 1L/min O_2 flow rate. Initially, all animals were pretested, in order to determine their hearing thresholds without any treatment. They were then tested again on the third day after cisplatin (Day 3), and a subset was tested on final day of the oral gavages (Day 6). Measurements made after cisplatin (Day 3 and Day 6) were compared to pre-exposure measurements to determine the extent of functional damage from the cisplatin exposure.

ABR testing

During testing, subcutaneous needle recording electrodes were placed at the vertex (non-inverting), below the left pinna (inverting), and behind the shoulder blade (ground) (Bielefeld, 2013). For ABR, test stimuli consisted of alternating phase tone bursts at the six determined frequencies. Signals were generated using Tucker Davis Technologies (TDT, Gainesville, Florida, USA) SigGen software. Each tone burst (1ms duration) was gated through a Blackmann window, and had a 0.5ms rise/fall time with no plateau. Stimuli were presented at a rate of 21/s and signals were routed to speaker (TDT model MF1) positioned at 0°azimuth, 17cm from the vertex of each rat's head. Acoustic stimuli were calibrated before each testing session by recording the output of the speaker with a microphone placed at the animals' head level. The rats' evoked responses were amplified

with a gain of 50000, using a TDT Headstage-4 bioamplifier, and bandpass filtered from 300 to 3000Hz. A total of 250 sweeps were averaged at each stimulus level using TDT BioSig software. The level of the signal was decreased in 5dB steps from 90dB SPL to a level 15dB below that of the lowest level (minimum level was 5 dB SPL) that induced a detectable and repeatable response. Threshold was recorded as the lowest level at which a detectable response was elicited and could be repeated.

DPOAE testing

In order to test the integrity of the OHC, cubic DPOAE were tested in the frequency range of 5-12 kHz in the rats. With the animals anesthetized with inhalant isoflurane (rats were still anesthetized from the ABR test), 6 mm rubber probes were placed in the animals' ear canals. DPOAEs were obtained using the TDT equipment described above. Stimuli were presented at 60 dB SPL and presented in descending 10-dB steps down to 20 dB SPL, with the L1/L2 ratio set at 1.0. Four f2 frequencies were tested: 5450, 7630, 10,900, and 13,080 Hz, and the f2/f1 ratio was 1.2. Noise floor measurements were collected for each ear, and the 2f1 - f2 DPOAE amplitudes of each sample was measured.

Cisplatin exposures

Following pre-testing for ABR and DPAOEs, all sixteen animals were administered the nine days of oral gavages, with the 12mg/kg cisplatin on the third day of the gavages. Cisplatin was delivered by intraperitoneal infusion. Animals were anesthetized, and a butterfly needle was inserted into the abdomen. The infusion was performed with syringe pusher (Kent Scientific) set at a volume of 8 mL/hour. Since the cisplatin was mixed at 1 mg/mL and the typical rat weighed 150-200 grams, the infusion typically took 12-16 minutes. For the week after the cisplatin exposure, the animals received their respective

gavage treatments every day, with testing of the ABR and DPOAEs performed on all animals on Day 3 and a subset of animals on Day 6. Animals were weighed daily and given 3 mL of saline sub-cutaneously every day to combat the dehydration that results from cisplatin toxicity.

Results

ABR Pretest results

ABR pretest thresholds are displayed in Figure 1. Both the control (blue) and saracatinib (red) rats had similar mean thresholds during pretesting. At 5 kHz, both groups had an average threshold of 20 dB SPL and at 40 kHz both thresholds were at 40 dB SPL. Two-factor analysis of variance (ANOVA) (frequency x group) revealed no significant interaction or main effect of group. Frequency was significant, but that was expected due to the higher pretest thresholds at 30 and 40 kHz. Since there were no statistically significant differences between both groups, these results served as a comparison level once the animals were exposed to the drugs.

ABR Day 3 Results

Mean ABR thresholds for the treated group and the control group on day 3 after the cisplatin exposure are displayed in Figure 2. On day 3, the mean thresholds of the control group were slightly lower than the thresholds of the experimental group. At higher frequencies, such as 30 kHz, the controls average threshold increased from the pretest threshold of ~40 dBSPL to ~70 dBSPL (a 30 dB increase), whereas the saracatinib average threshold increased from ~40 dBSPL to ~80 dBSPL (a 40 dB increase). Although there are differences between the means of the two groups on day three, the three-factor repeated

measures ANOVA (frequency x group x test day) revealed that differences were not statistically significant.

ABR Day 6 Results

Mean ABR thresholds for the treated group and the control group on day 6 after the cisplatin exposure are displayed in Figure 3. On day 6, the average thresholds in the experimental group were lower thresholds than those in the control group. The control group increased from a pretested average threshold of 40 dBSPL to 90 dBSPL by day 6 (a 50 dB increase), whereas the experimental group increased from 40 dBSPL to 70 dBSPL (a 30 dB increase). Note by day 6, only three of the eight rats from the saracatinib group were tested and four of the eight from the control were tested. Although there is some indication that saracatinib was protecting the ears from damage, the results from day six were not statistically significant.

DPOAE Results

Distortion product otoacoustic emissions were tested at four different frequencies (5, 7, 10, and 12 kHz). Results are displayed in Figures 4-7. Amplitudes were expressed as nV about the noise floor. A three factor ANOVA (group x stimulus level x test day) was used to evaluate changes in amplitudes pre- and post-cisplatin in the treated and control groups. Each frequency was analyzed separately. At all four frequencies, saracatinib (blue) and control (red) subjects were not statistically different from each other prior to cisplatin exposure. At day 3, after cisplatin exposure, both saracatinib (green) and control (purple) rats' amplitudes were significantly decreased from pre-exposure levels, but the groups were not different from each other. If saracatinib protected the OHCs from damage, the

saracatinib group on day three (green) would have had similar amplitudes to the pretest results (blue), and would have been higher than the control group.

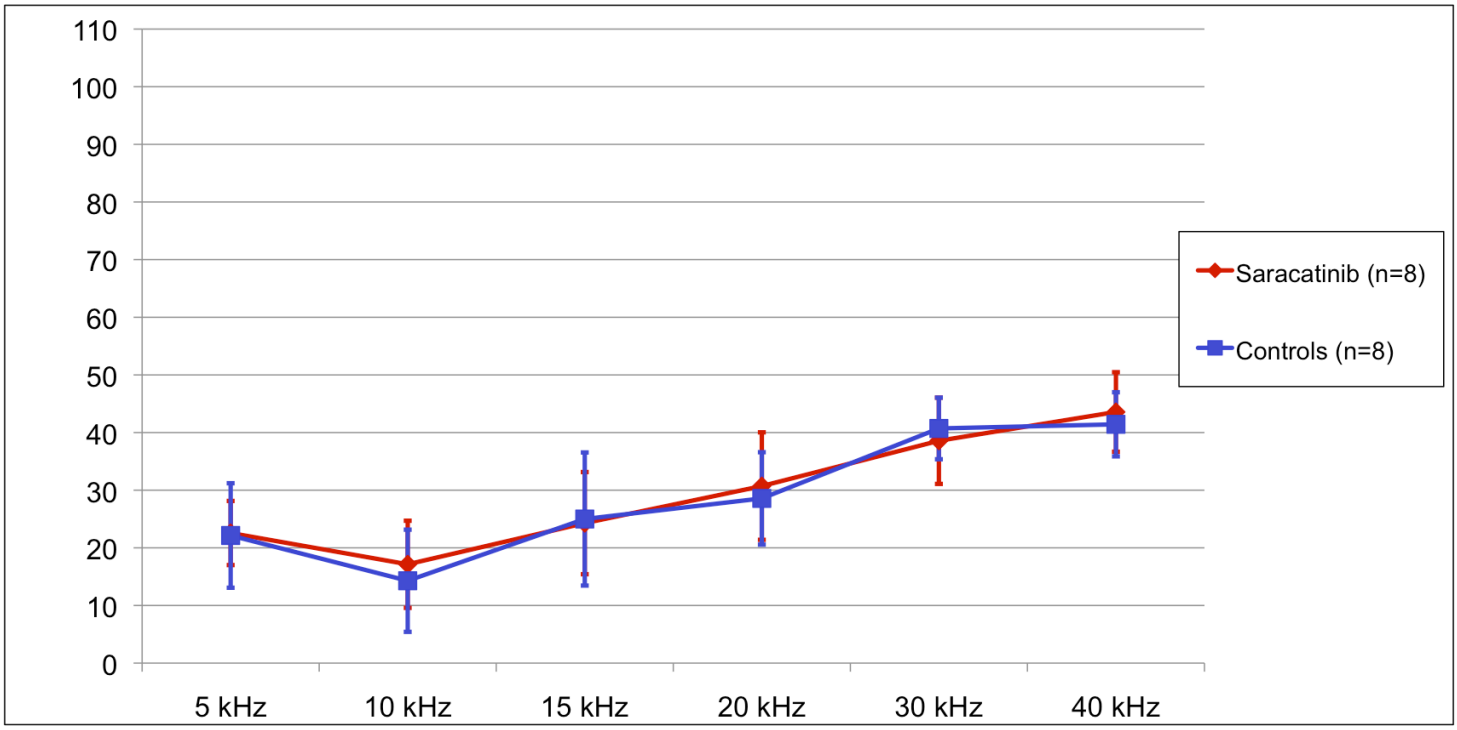


Figure 1: ABR threshold averages: pretest results

Red: Animals exposed to saracatinib and cisplatin (experiments)
Blue: Animals exposed to vehicle and cisplatin (controls)
No statistically significant differences were detected.

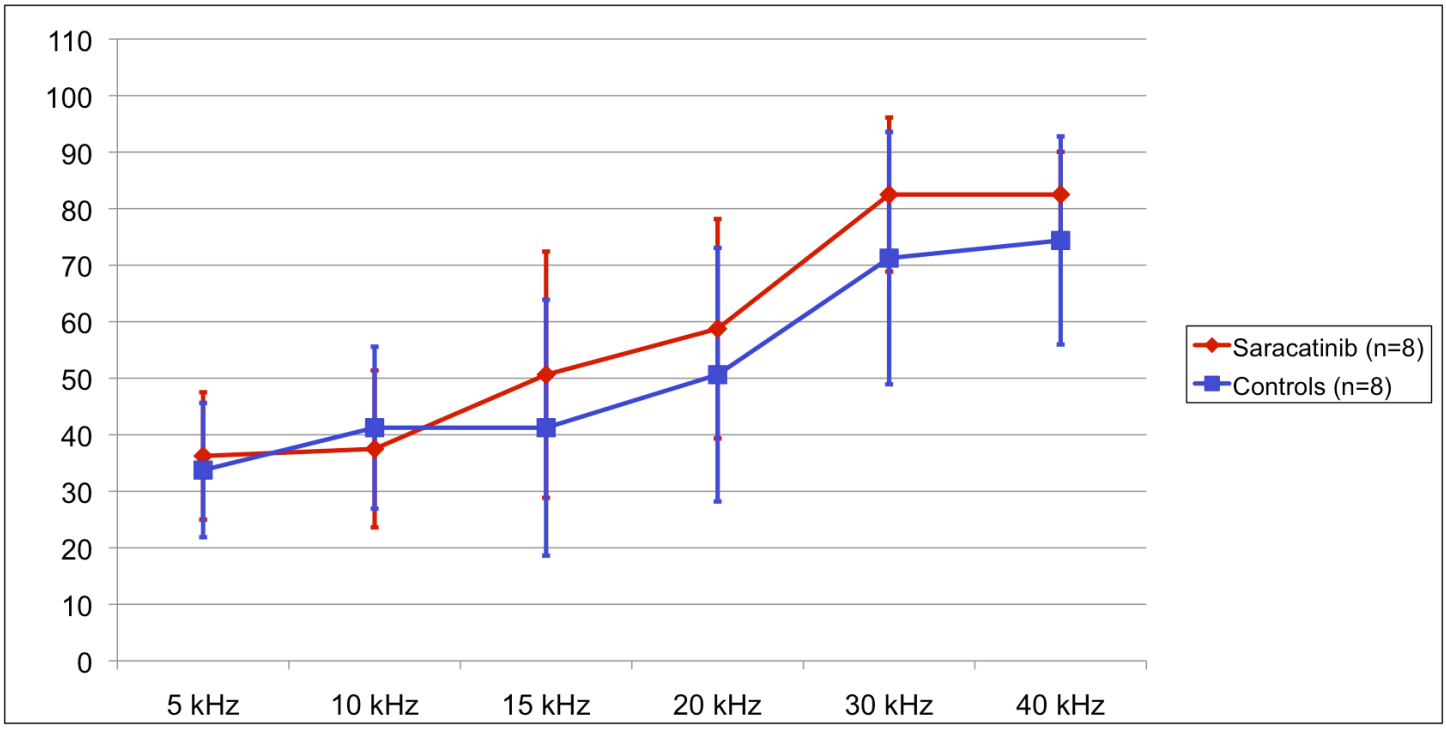
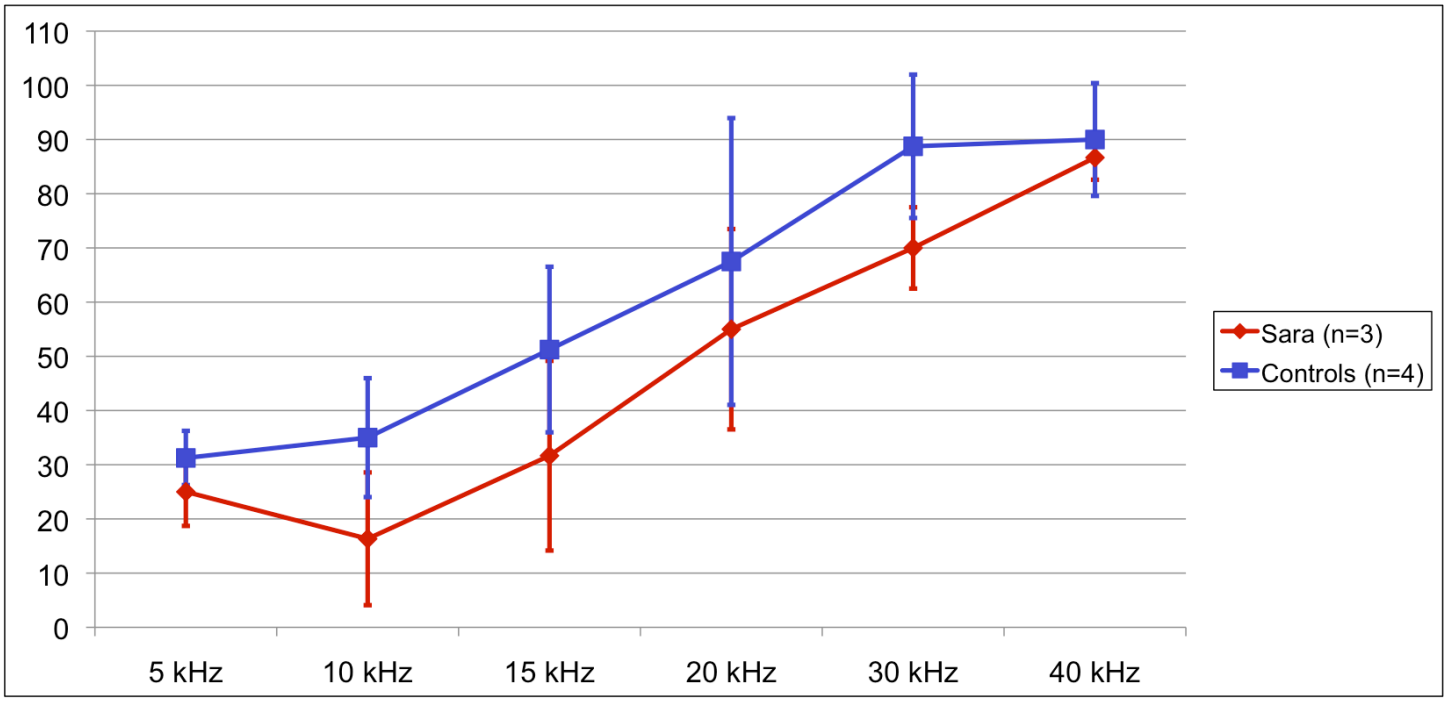


Figure 2: ABR threshold averages: Day 3 results

Red: Animals exposed to Saracatinib and cisplatin (experiments)
Blue: Animals only exposed to cisplatin (controls)
On Day 3, the mean thresholds of the control group were slightly lower than the thresholds of the experimental group, but differences were not statistically significant.



<u>Figure 3</u> ABR threshold averages: Day 6 results

Red: Animals exposed to Saracatinib and cisplatin (experiments)
Blue: Animals only exposed to cisplatin (controls)
Note: only 3 Saracatinib rats and 4 control rats were tested at Day 6.
On Day 6, the mean thresholds in the experimental group were lower thresholds than those in the control

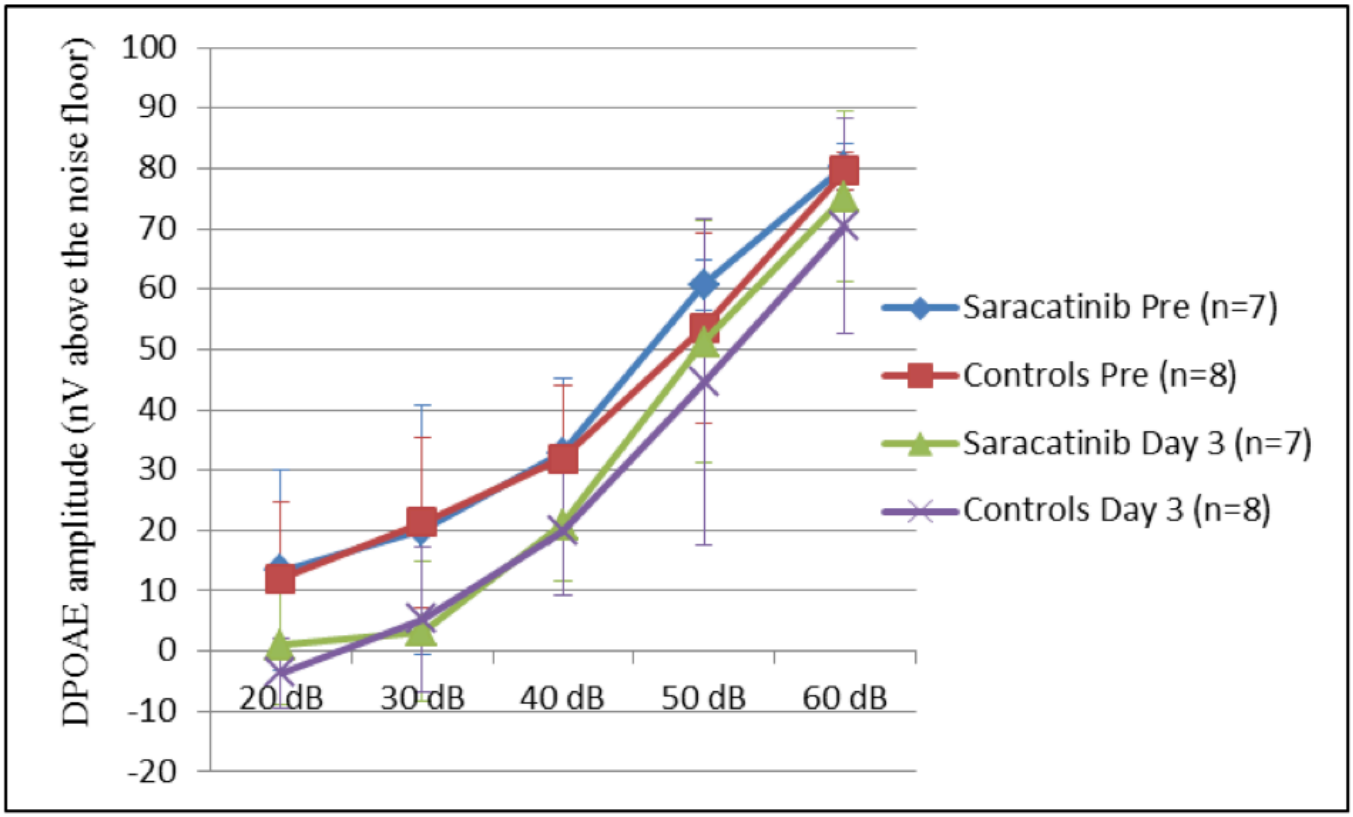


Figure 4: DPOAE Amplitude at 5KhZ

Amplitudes are expressed as nV above the noise floor. Saracatinib (blue) and control (red) rats were not statistically different from each prior to the cisplatin exposure. At Day 3 after cisplatin, both the Saracatinib (green) and control (purple) rats' amplitudes were significantly decreased from pre-exposure levels, but the groups were not different from each other at Day 3.

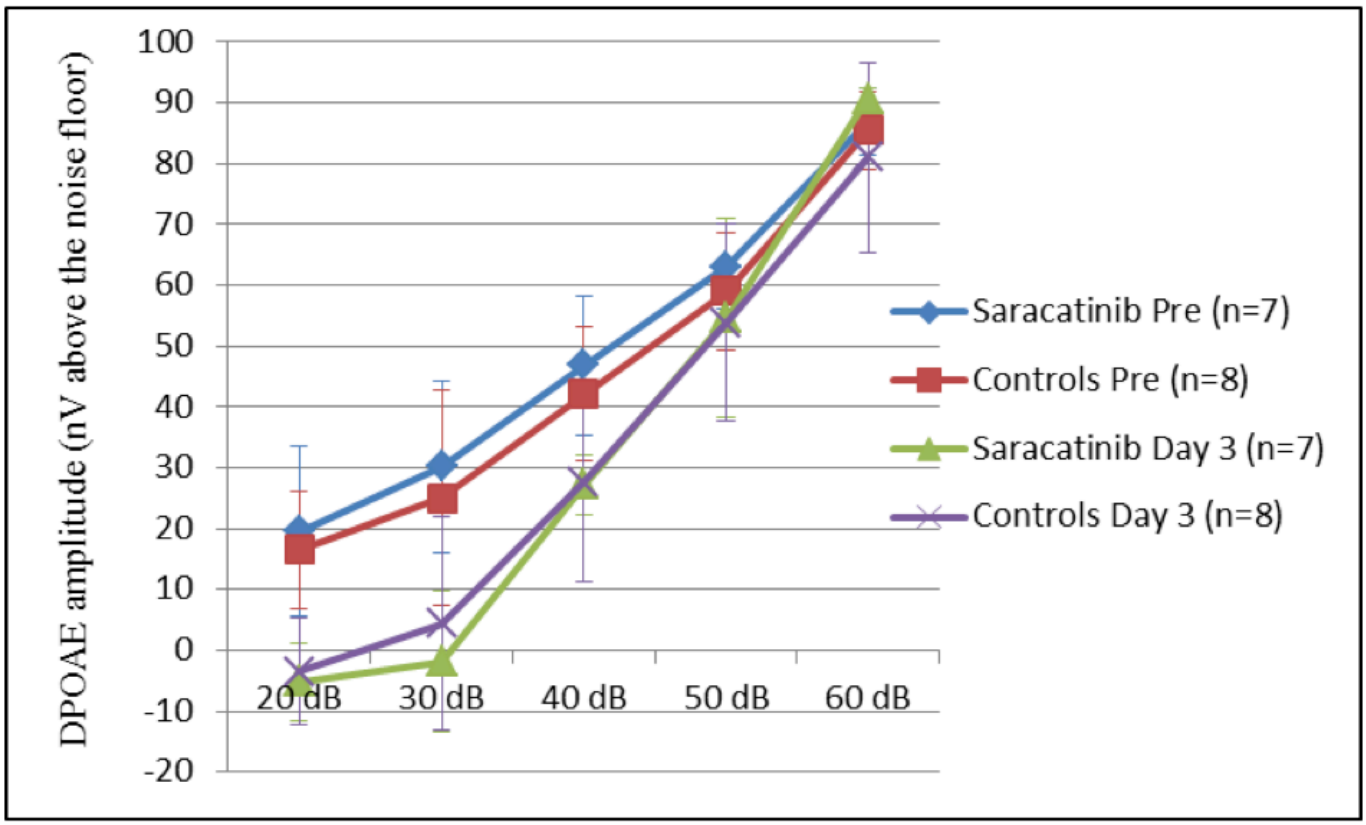


Figure 5: DPOAE Amplitude at 7KhZ

Amplitudes are expressed as nV above the noise floor. Saracatinib (blue) and control (red) rats were not statistically different from each prior to the cisplatin exposure. At Day 3 after cisplatin, both the Saracatinib (green) and control (purple) rats' amplitudes were significantly decreased from pre-exposure levels, but the groups were not different from each other at Day 3.

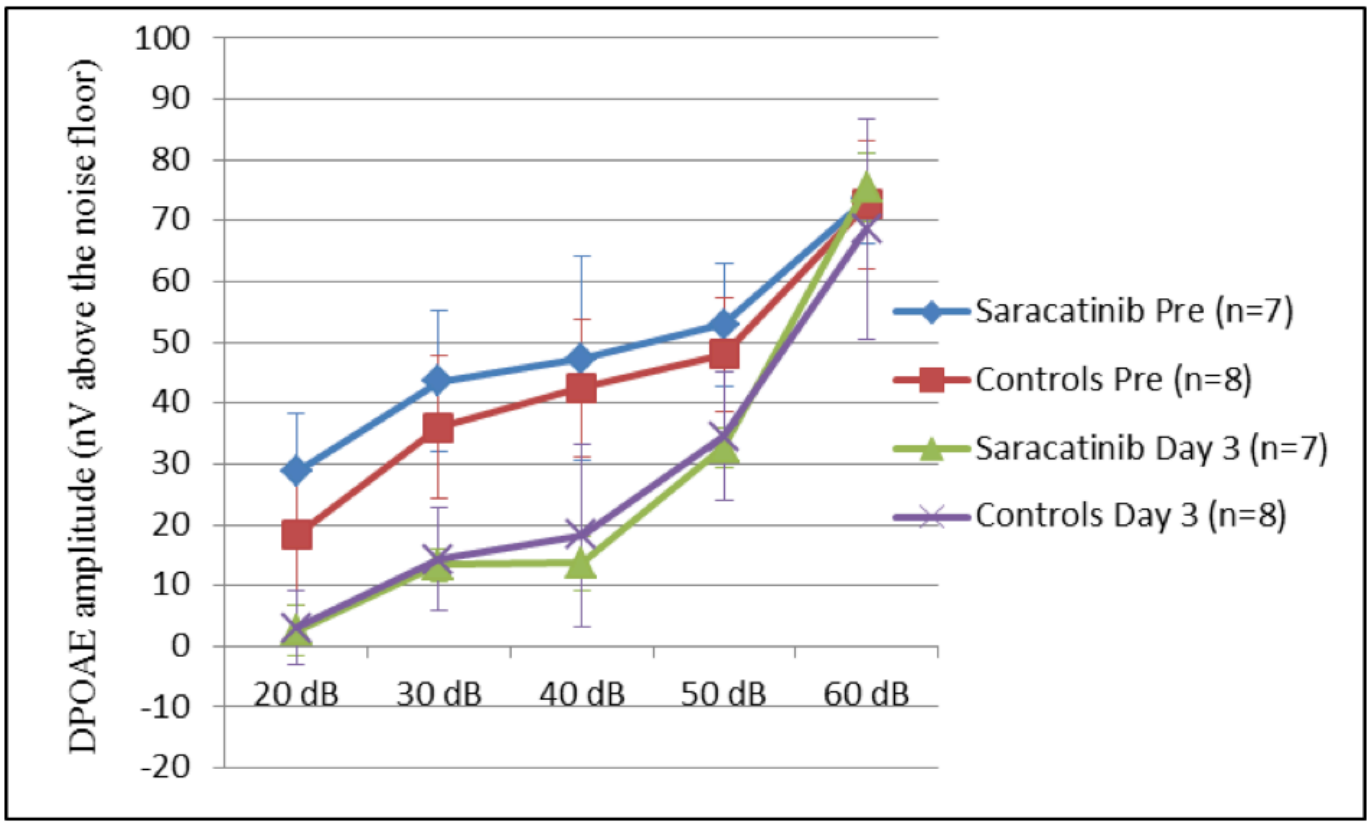


Figure 6: DPOAE Amplitude at 10KhZ

Amplitudes are expressed as nV above the noise floor. Saracatinib (blue) and control (red) rats were not statistically different from each prior to the cisplatin exposure. At Day 3 after cisplatin, both the Saracatinib (green) and control (purple) rats' amplitudes were significantly decreased from pre-exposure levels, but the groups were not different from each other at Day 3.

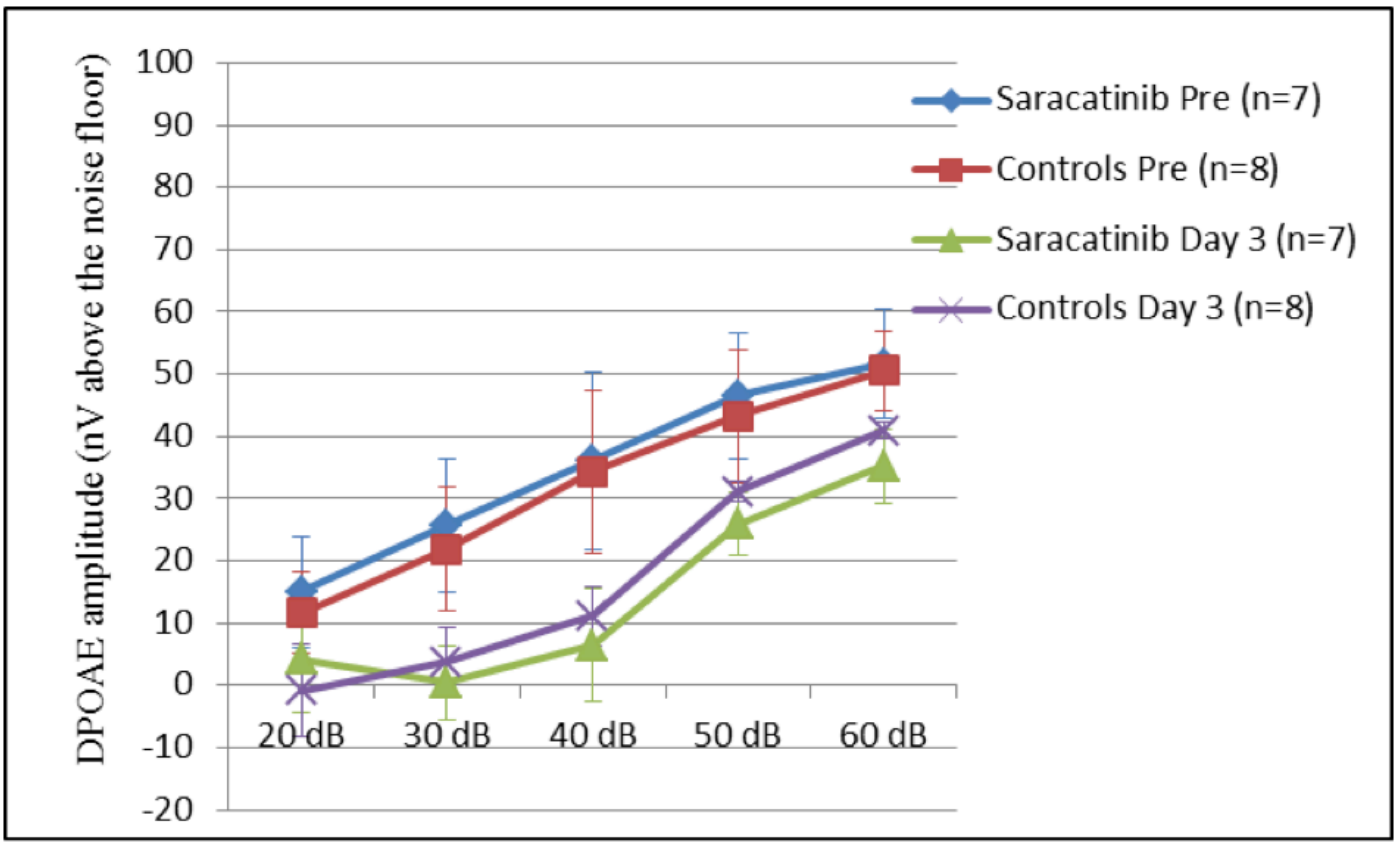


Figure 7: DPOAE Amplitude at 12KhZ

Amplitudes are expressed as nV above the noise floor. Saracatinib (blue) and control (red) rats were not statistically different from each prior to the cisplatin exposure. At Day 3 after cisplatin, both the Saracatinib (green) and control (purple) rats' amplitudes were significantly decreased from pre-exposure levels, but the groups were not different from each other at Day 3.

Discussion

There was no statistically significant difference for the ABR pretest results between the control and experimental groups. On day 3 of ABR testing, the average thresholds of the control group were slightly lower than those of the experimental group. Although this contradicts the hypothesis, the results were not statistically significant. The average thresholds of the experimental group were lower than the thresholds of the control group on day 6 of ABR testing. Since only 3 experimental rats and 4 control rats were tested and there was only a minor difference between thresholds, the results were not statistically significant. DPOAE results at all four amplitudes had no statistically significant differences between pre-exposure and Day 3 for both the saracatinib and control rats.

The lack of significance at Day 6 can potentially be attributed to lack of statistical power due to the small number of animals tested at that time point. Clearly, the compound was not effective in protecting hearing at the Day 3 time point. If more rats were tested through Day 6, the results would have been more easily interpreted for determining if the compound could prevent hearing loss and OHC damage after cisplatin exposure.

With protection studies, a negative result can occur for a number of reasons: the dose may have been too high or low, the oral route of administration may have left too little compound to enter the cochlea, the timing of the doses may have been poor, or the compound simply may not be effective. As noted earlier, saracatinib is designed to prevent Src-mediated apoptosis and act as a blocker of OCT. It is possible that the compound was not successful in preventing apoptosis in the OHCs and the cells were still damaged. It is also possible that the Src pathway involved in cisplatin ototoxicity is part of more complex sequence of cell signaling. Interrupting the Src signaling pathway on the time schedule that

was used in the current study may have led damage pathways that offset the protective value of the Src inhibition. Another possibility is that the compound was not effective in blocking OCT, and cisplatin was still entering the cells in the cochlea. OCT in the cochlea is a relatively new area of investigation, and the ability to block OCT in the cochlea pharmaceutically is a challenge due to the limitations imposed by the blood-labyrinth barrier that restricts pharmaceutical penetration into the cochlea. Future studies are needed to address these issues.

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